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Production of Living Nanoparticles for Blood Cancer Therapy

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UNDER MY SUPERVISION BY

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ENTITLED

PRODUCTION OF LIVING NANOPARTICLES FOR BLOOD CANCER THERAPY

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PRODUCTION OF LIVING NANOPARTICLES FOR BLOOD CANCER THERAPY

by

Michael Pierotti, Matthew Piro, and Peter Mitchell

SENIOR DESIGN PROJECT REPORT

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Abstract

Current cancer therapies leave much to be desired because they are very harmful to the patient and cause a significant decrease in quality of life. Chimeric Antigen Receptors (CAR) are a promising novel approach for treating specific types of leukemia due to their binding affinity for proteins expressed solely on leukemia B cells. This approach increases specificity of how cells receive treatment, thus allowing for the destruction of cancerous cells while leaving the healthy cells unharmed. In this experiment, we show that production of CAR expressing exosomes (liposome like vesicles produced naturally by human cells) is possible through cell transfection. This finding demonstrates that a new wave of cancer therapeutics, that are more specific and have less harmful side effects, are producible.

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List of Abbreviations

CAR: Chimeric Antigen Receptor

DAPI: 4′,6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Media

DNA: Deoxyribonucleic acid

FDA: Food and Drug Administration

GFP: Green Fluorescent Protein

HEK: Human Embryonic Kidney cells

PEI: Polyethylenimine

PBS: Phosphate Buffered Saline

tVSVG: Truncated vesicular stomatitis virus glycoprotein (exosomal membrane protein)

Introduction

Background

Current cancer therapies are plagued by low success rates and a multitude of side effects that make the treatment agonizing for the patient. Targeted attack of cancer cells is thought to result in fewer side effects when compared to treatments that indiscriminately kill both healthy and cancerous cells. New research has proven that the Chimeric Antigen Receptor (CAR) can effectively target and bind to leukemia B-cells. In our experiment we utilize exosomes, extracellular vesicles derived from the membrane of their host cells, to incorporate a CAR protein into the exosomal membrane. The CAR-expressing exosome can potentially be employed as a targeted drug-delivery vehicle to provide therapy for leukemia cells. We believe that CAR-expressing exosomes will be able to efficiently bind to specific cancer cells, thus allowing for more targeted therapy, and significantly reducing the side effects of other cancer treatments.

Review of Field

One of the main types of nanoparticles used in research is living nanoparticles. Living nanoparticles are naturally produced in the human body. The most popular example is liposomes, which are spherical vesicles made from lipids that are used in cell-cell communication. Currently, there are several FDA approved nanoparticle-assisted delivery agents for cancer therapy, and only one of which does not utilize liposomes as a delivery agent. The first nanoparticle delivery drug to be approved is Doxil, which received FDA approval in 1995, while the most recent is Vyxeos, which was approved in August of 2017[1]. The only non-liposomal based nanoparticle delivery system is Abraxane, which is an albumin-bound paclitaxel nanoparticle. Although these drugs have been approved as cancer therapies, all of them use passive targeting, rather than the more effective active targeting. Passive targeting of tumors is mainly achieved through the

[1] Anselmo, Aaron C., and Samir Mitragotri. "Nanoparticles in the Clinic." *Bioengineering & Translational Medicine*, vol. 1, no. 1, 2016, pp. 10–29., doi:10.1002/btm2.10003.

enhanced permeability and retention (EPR) effect, which means the drug tends to accumulate in areas of low vascularization, such as tumors. Active targeting is based on the technology to attach specific ligands to the surface of a drug delivery nanoparticle, which will then attach and bind to specific cancer cells[2].

Another category of nanoparticles being explored for targeted therapies are non-living nanoparticles, which are not naturally produced in the human body. The two most promising nanoparticles in this category are gold and titanium dioxide. Gold nanoparticles are being researched as drug delivery systems as well as other potential applications, like photothermal agents, contrast agents, and radiosensitisers[3]. Titanium dioxide nanoparticles are also being explored as drug delivery agents, and are being researched in vivo as well as in vitro[4].

Critique of Current Technology

Although liposomes are naturally occurring, they do not naturally express membrane proteins. Thus, attaching antibodies to the surface of these nanoparticles is inherently more difficult. The increased difficulty of attaching ligands to the surface of these nanoparticles is the main reason why none of the FDA approved liposome drug delivery systems use active targeting, even though active targeting has been shown to be more effective for cancer treatment in preclinical studies[5]. Although there have been studies looking at targeted therapy using liposomes expressing monoclonal antibodies on their surface, all of these potential therapies have failed, in both efficacy as well as toxicity (mostly immunogenicity)[6]. Additionally, liposomes are one of the larger nanoparticles, which leads to difficulty passing blood barriers like the blood-brain barrier. While some limited advances have been made in crossing the blood brain barrier using

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[2] Torchilin, Vladimir P. "Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example." *Drug Delivery Handbook of Experimental Pharmacology*, 2009, pp. 3–53., doi:10.1007/978-3-642-00477-3_1.

[3] Jain, S, et al. "Gold Nanoparticles as Novel Agents for Cancer Therapy." *The British Journal of Radiology*, vol. 85, no. 1010, 2012, pp. 101–113., doi:10.1259/bjr/59448833.

[4] Bogdan, Janusz, et al. "Nanoparticles of Titanium and Zinc Oxides as Novel Agents in Tumor Treatment: a Review." *Nanoscale Research Letters*, vol. 12, no. 1, 2017, doi:10.1186/s11671-017-2007-y.

[5] Anselmo, Aaron C., and Samir Mitragotri. "Nanoparticles in the Clinic." *Bioengineering & Translational Medicine*, vol. 1, no. 1, 2016, pp. 10–29., doi:10.1002/btm2.10003.

[6] Sercombe, Lisa, et al. "Advances and Challenges of Liposome Assisted Drug Delivery."*Frontiers in Pharmacology*, vol. 6, Jan. 2015, doi:10.3389/fphar.2015.00286.

membrane-bound proteins on liposomes, these have also failed due to the poor pharmacokinetics and immunogenicity mentioned above[7].

Gold and titanium dioxide nanoparticles are not naturally occuring in the body, which means there are higher risks of toxicity. Both of these nanoparticles have been shown to be toxic to humans in potential therapeutic doses during in vivo studies[8,9]. Neither of these particles have the ability to express antibodies or other membrane proteins, which means that they must rely on passive targeting through EPR or other mechanisms, instead of the more effective active targeting. Additionally, there are environmental concerns with both of these nanoparticles. Since they do not degrade, they tend to accumulate in tissues, which causes toxicity in humans as well as bioaccumulation in the natural world during their manufacturing[10]. All of these risks and challenges to using non-living nanoparticles are a major roadblock to their acceptance as a drug-delivery agent by the FDA.

Project Goal

Our aim is to avoid the problems of other nanoparticles by using naturally secreted exosomes as vehicles for targeted delivery. In order to target leukemia cells, we will integrate the Chimeric Antigen Receptor (CAR) into the exosome membrane. CAR is composed of three different proteins as depicted in Figure 1. scFV is a single chain antibody that has a high binding affinity for leukemia cells and drives the exosomes towards the cancerous cell. tVSVG is a truncated membrane protein which allows for the components of CAR to attach to both the inside and outside of the exosome. This protein ensures that the CAR protein will be expressed on the exosome membrane and nowhere else. Green Fluorescent Protein (GFP) serves as a marker to allow us to differentiate between exosomes expressing the CAR protein and exosomes without the CAR protein. In order to engineer cells that produce exosomes with the CAR protein, we must integrate

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[7] Vieira, Débora B, and Lionel Gamarra. “Getting into the Brain: Liposome-Based Strategies for Effective Drug Delivery across the Blood&Ndash;Brain Barrier.”*International Journal of Nanomedicine*, Volume 11, 2016, pp. 5381–5414., doi:10.2147/ijn.s117210.

[8] Shi, Hongbo, et al. “Titanium Dioxide Nanoparticles: a Review of Current Toxicological Data.” *Particle and Fibre Toxicology*, vol. 10, no. 1, 2013, p. 15., doi:10.1186/1743-8977-10-15.

[9] Lopez-Chaves, Carlos, et al. “Gold Nanoparticles: Distribution, Bioaccumulation and Toxicity. In Vitro and in Vivo Studies.” *Nanomedicine: Nanotechnology, Biology and Medicine*, 2017, doi:10.1016/j.nano.2017.08.011.

[10] Shah, Syed Niaz Ali, et al. “Hazardous Effects of Titanium Dioxide Nanoparticles in Ecosystem.” *Bioinorganic Chemistry and Applications*, vol. 2017, 2017, pp. 1–12., doi:10.1155/2017/4101735.

the CAR DNA into the cell's genome using transfection. Transfection is the process of deliberately introducing genetic material into the cells and allowing the cells to naturally uptake the DNA. If successful, we plan to observe engineered exosomes that express the CAR protein and can effectively bind to leukemia B-cells.

Backup Plan

Our backup plan was to purchase professionally prepared lentiviruses that have the desired DNA inside of them. We could then integrate the CAR DNA into the genome of the cell using lentiviral transduction. This plan is very likely to work because it does not involve much experimentation on our part. We would simply be adding the lentiviruses into the dish with cells and determining if the desired gene had been expressed. The main reason that this option is our backup is because it is very expensive[11] and we are already using nearly all of our allocated money on our first experimental design, so we would not be able to afford this without applying for extra financial support.

Significance

We believe that our technology will have a large impact on the future of cancer therapies. We currently have two ideas about how this technology will be built upon in the future. The first possibility is that the same model that we are using will be adapted to contain a toxic protein rather than GFP so that when the exosomes bind to leukemia cells they will deposit that protein, leading to the death of the cell. The second possibility is that the single chain antibody could be changed to target other types of disease states. That means that this technology could potentially be adapted for treatment of a wide range of diseases.

Team and Project Management

Table 1: Team Members and Faculty Advisor

[11] Inc., Applied Biological Materials. "Custom Lentivirus." *Custom Lentivirus*, www.abmgood.com/Custom-Lentivirus-Subcloning-Services.html.

Team Members		
Michael Pierotti*	Biomolecular Engineering	mpierotti@scu.edu
Matthew Piro	Pre-Med Bioengineering	mpiro@scu.edu
Pete Mitchell	Biomolecular Engineering	psmitchell@scu.edu
Faculty Advisor		
Bill Lu (MD, PhD)	School of Bioengineering	blu2@scu.edu

* Denotes primary contact

Management Approach

An excerpt from our team charter effectively displays our management style throughout our project:

"We will all be responsible for a portion of each milestone or objective, and the portion that each individual will be responsible for every week will be assigned by the weekly team leader, a role that switches from student to student each week. Our main expectations are that everyone will be professional in communications, all work will be completed on time, and all work will be held to our usual standard and the standard of Dr. Lu."

Budget and Issues

Our full project budget can be viewed in Appendix D. Our experiment is currently operating within our budget constraints. However, since we only have $110 in reserve, we do not have much room for error. The only upcoming part of the experiment which might force us to use this reserve budget would be sending a sample of our exosomes out to a company in order for them to test the concentration. Since we are unsure whether we will end up doing this supplement to our testing protocol, we may not use the last of our budget reserves.

Timeline and Issues

Table 2: Timeline

	Fall Quarter	**Winter Quarter**	**Spring Quarter**
Weeks 1-3	Safety training, create outline for the year	Run experiments	Finish experiments (if necessary), create + finish powerpoint
Weeks 4-7	Cell culture technique practice, budget proposal, finalize experiment procedure	Run experiments	Create draft of presentation script, continue writing report
Weeks 8-10	Kill cells before break, write introduction section of paper	Run experiments, compile data	Finish presentation script + written report, practice presentation, final presentation and report

Note: Gantt charts with further information can be seen in Appendix B.

Chapters of Project

The goals we have for this project are stratified in order of importance, due to the unpredictable nature of working with living cells. Our baseline goal was to successfully transfect the HEK 293 cells with the genes coding for the CAR protein. We could verify this through simple fluorescent microscopy. If our cells are glowing green, we know that the GFP gene is being transcribed. We could further verify successful incorporation into the cells genome by introducing the cells into media with added puromycin. Since the gene we added encoded for puromycin resistance, only the cells that had incorporated the gene into their genome would survive. This was our baseline goal to prove that we could successfully transfect cells so they could generate exosomes with the CAR protein.

Details of Key Constraints

We have two main constraints for our project. The first is to ensure the transfection of our DNA is successful. We can validate this through viewing of the cells through a fluorescent microscope to check for GFP, as well as introducing the cells to media with added puromycin to check if they express the puromycin resistance gene introduced through the CAR DNA. The next key constraint is to ensure that the cells actually produce exosomes. Although background research states that they will, we will conduct an exosome harvest to validate this. We will also obtain the concentration to know the production rate for our exosomes to prove that the transfection does not interfere with this efficiency.

Detailed Design Description

The exosome that we are engineering is depicted in Figure 1 of the appendix. The main features of this nanoparticle are the CAR-T single chain antibody, the truncated VSVG membrane protein, and the green fluorescent protein (GFP) housed within the body of the exosome. The CAR-T single chain antibody is the protein that has a high binding affinity for a receptor on Leukemia B cells. The truncated VSVG membrane protein ensures that this complex will be localized to exosomes because it is only present

in the membranes of exosomes. The GFP that is housed within the exosome functions as a tag so that we can easily see if the cells are expressing this protein complex by detecting the green fluorescence using fluorescence microscopy.

To create our desired exosome we are going to transfect HEK 293 cells with the DNA vector shown in Figure 2. This vector codes for the protein complex to be created and has a few other functionalities as well. It includes a promoter (MSCV) to drive the production of our protein, a secondary promoter (EF1) that will increase the cells puromycin resistance expression (Puro) along with a poly A tail for stability.

Detailed Supporting Analyses

We will examine the concentrations of nanoparticles (exosomes) produced, as well as testing their ability to be uptaken by control cells.

Expected Results

The results are mainly focused on determining if exosomes that have a binding affinity for leukemia B cells can be produced. The binding affinity of CAR-T has already been proven[12], so the experiment will be focused on production levels of exosomes. The data will be comparing the concentration of nanoparticles/ml to other concentrations of exosomes and liposomes produced for therapeutic purposes. We also expect that there will be uptake of the exosomes into the control cells.

[12] Arcangeli, Silvia et al. “Balance of Anti-CD123 Chimeric Antigen Receptor Binding Affinity and Density for the Targeting of Acute Myeloid Leukemia” Molecular Therapy , Volume 25 , Issue 8 , 1933 - 1945

Materials and Methods

Passaging HEK 293 Cells

Check cells under the microscope. They should be passaged at 70-80% confluency. Warm cell media (DMEM), PBS, and trypsin in a 37°C water bath. Then move the cells, DMEM, PBS, trypsin, aspirating tips, pipet tips, pipetman, and P1000 micropipette to the hood. Aspirate the old media out of the well plate. Add 3 ml of PBS to the plate, aspirate out the PBS, and add 1.5 ml of Trypsin dropwise throughout the plate. Put the plate in the incubator for 2 minutes. Add 5 ml of DMEM to the plate. Pipet contents of the plate into a microcentrifuge tube. Centrifuge the tube with for 5 minutes at 1500 rpm. Label the new plate with initials, cell type, passage number, and date. Aspirate out the liquid in the tube. Do not aspirate out the cell pellet. Add 7 ml of DMEM to the new 60mm plate, resuspend cell pellet in 4 ml of DMEM, and micropipet 250 μl from the centrifuge tube to the new plate.

Transfection of HEK Cells

First, add 300 μl of OptiMem to 2 Microcentrifuge tubes and label them "DNA" and "Transfection Reagent". Next add 6ug of desired DNA to the tube labeled DNA. Then add 30 μl of PEI to the tube labeled "Transfection Reagent" and let rest for 10 minutes. Transfer all contents of the "DNA" tube into the "Transfection Reagent" tube and gently mix, wait 20 minutes. Finally, plate 200 μl into each well and swirl gently. For control solution, repeat same steps as listed above without adding the DNA.

Passaging HEK 293 Cells (From 6-well Plate onto 60 mm Plate)

Check cells under the microscope. Cells should be passaged at 70-80% confluency. Warm cell media (DMEM), PBS, and trypsin in a 37°C water bath. Move cells, DMEM, PBS, trypsin, aspirating tips, pipet tips, pipetman, and P1000 micropipette to the hood. Aspirate the old media out of the well plate, add 1.5 ml of PBS to the plate, aspirate out the PBS, and add 1 ml of Trypsin dropwise throughout the plate. Then put

the plate in the incubator for 2 minutes. Next, add 3 ml of DMEM to the plate, then pipet contents of the plate into a microcentrifuge tube. Centrifuge the tube with for 5 minutes at 1500 rpm. Label the new 60 mm plate with initials, cell type, passage number, and date. Aspirate out the liquid in the tube. Do not aspirate out the cell pellet. Add 3 ml of DMEM to the new plate, resuspend cell pellet in 2 ml of DMEM, and micropipet 250 µl from the centrifuge to the new normal-sized plate.

Passaging onto 100 mm Plates for Exosome Harvest

Check cells under the microscope. They should be passaged at 70-80% confluency. Warm cell media (DMEM), PBS, and trypsin in a 37°C water bath. Then move the cells, DMEM, PBS, trypsin, aspirating tips, pipet tips, pipetman, and P1000 micropipette to the hood. Aspirate the old media out of the well plate. Add 3 ml of PBS to the plate, aspirate out the PBS, and add 1.5 ml of Trypsin dropwise throughout the plate. Put the plate in the incubator for 2 minutes. Add 5 ml of DMEM to the plate. Pipet contents of the plate into a microcentrifuge tube. Centrifuge the tube with for 5 minutes at 1500 rpm. Label the new plate with initials, cell type, passage number, and date. Aspirate out the liquid in the tube. Do not aspirate out the cell pellet. Add 20 ml of DMEM to the new 100 mm plate, resuspend cell pellet in 4 ml of DMEM, and micropipet 1.5 ml from the centrifuge tube to the new plate.

Exosome Harvest for Exosomes in Suspension on 100 mm Plates

After cells have been plated on 100 mm plates for one day, aspirate off the DMEM and add 20 ml of Ultraculture. Place back in incubator and leave for 2 days. After 2 days have passed remove media using a pipette and place in a 50 ml centrifuge tube. Centrifuge at 1500 x g for 10 minutes and then filter through a 0.2um filter. Divide the current volume by 4 and add that much EXO-TC to the tube. Leave in the 4°C fridge overnight. The next day centrifuge the exosome suspension at 3000 x g for 90 minutes. Remove the supernatant and let the tube dry, the exosomes will be in a pellet at the bottom. Resuspend the exosomes in 100 µl of PBS and place into a microcentrifuge tube.

Plating for Confocal Microscopy

During routine passaging of cells, once cells are resuspended in new media, plate 100 μl of the cell suspension onto a glass bottom plate with 1.9 ml DMEM. Wait two days for cells to attach to plate, then aspirate off DMEM, and add 2 ml of PBS.

DAPI Staining Procedure

Create DAPI solution of 300 nM by diluting as needed with PBS. Take control cells on glass bottom plate and wash 3 times with PBS. Aspirate off PBS and add enough DAPI solution to cover the cells. Incubate for 5 minutes, then aspirate off DAPI solution. Wash 3 times with PBS, and then take confocal microscopy pictures as necessary.

Exosome Uptake Experiment into Control HEK 293 Cells

After isolating exosomes, take exosomes and resuspend in 100 μl of PBS. Add exosome suspension into glass bottom plate with control HEK cells. Take confocal microscopy pictures as necessary.

Results

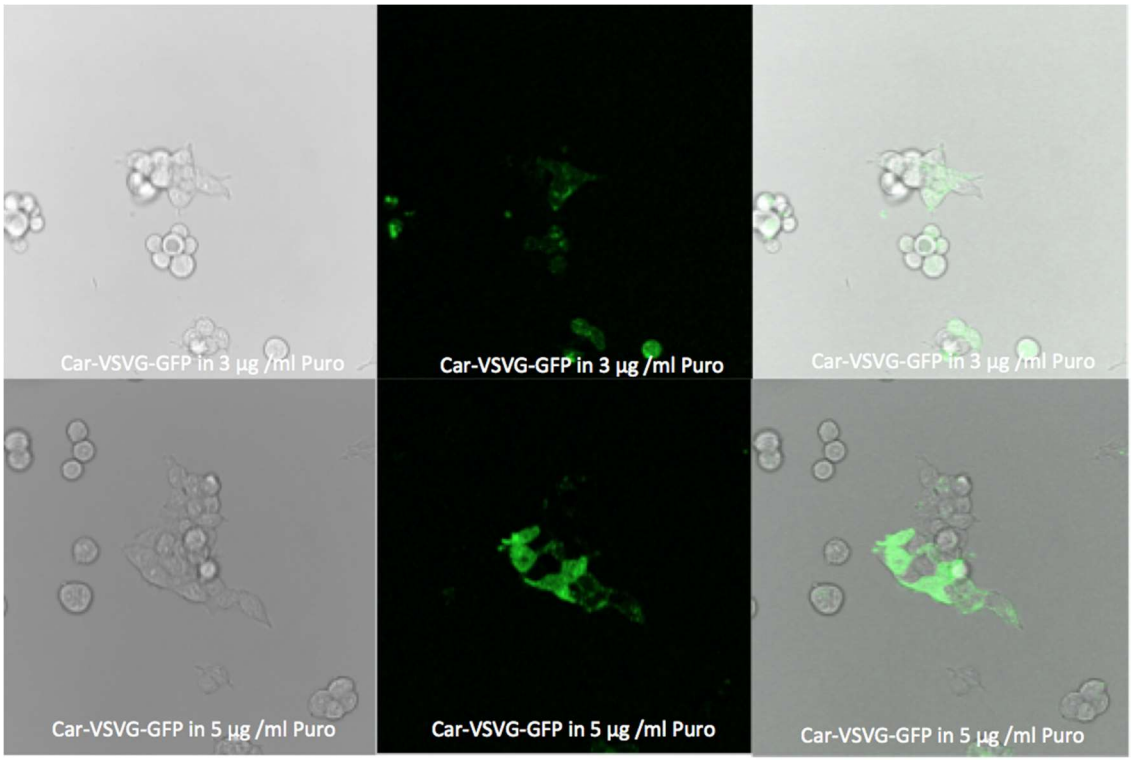

Figure 1: Experimental Cells 31 Days after Transfection. Figure 1 shows our experimental cells 31 days after transfection. Figure 1A,D show the experimental cell lines in phase lighting. 1B,E show the cells being exposed to green fluorescent light. 1C,F show the phase and green fluorescent light images overlapped for both of the conditions. As you can see there is GFP being expressed in both of the experimental conditions.

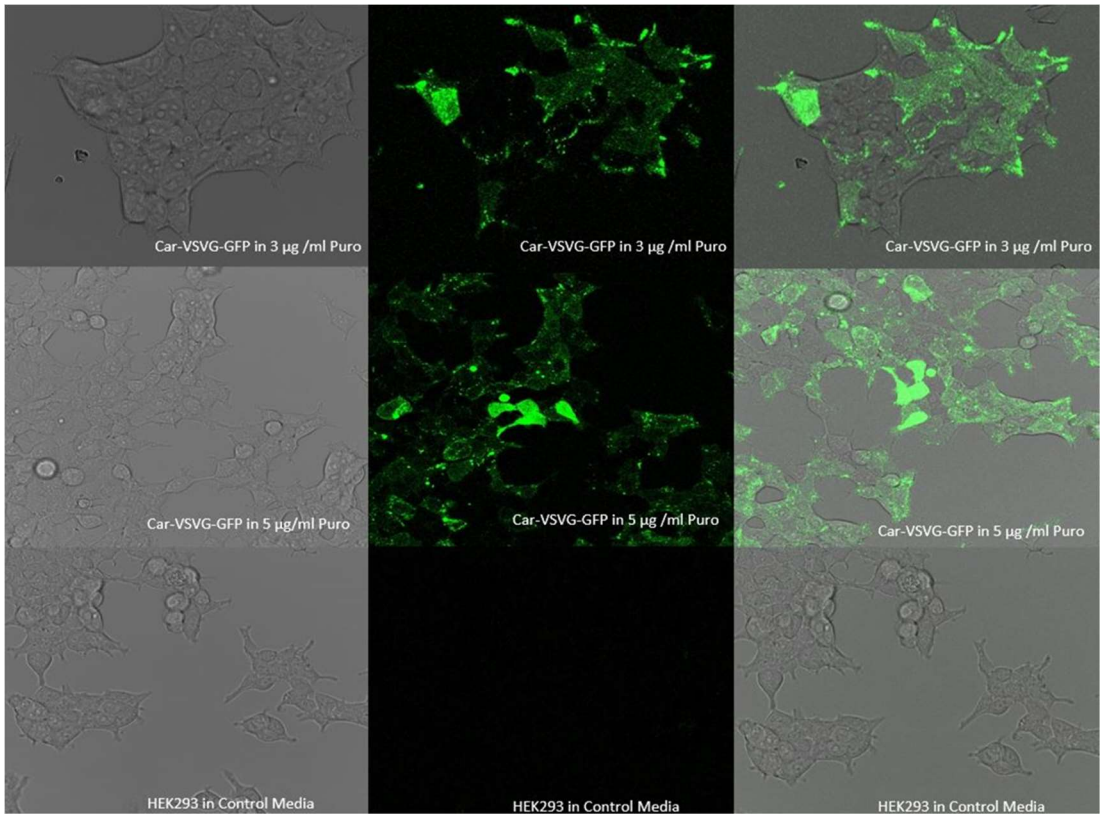

Figure 2: Cells 79 Days after Transfection: Figure 2 shows both experimental lines of cells and a control line of cells 79 days after transfection. 2A,G,D show all of the conditions in phase lighting. 2B,E,H show each condition under green fluorescent light. Finally, 2C,F,I show an overlay of the phase and green fluorescent light in each condition.

Figure 3: DAPI Staining of Experimental Cells Figure 3 shows two lines of experimental cells and a control line of cells exposed to green fluorescent and blue fluorescent light after they have been stained by DAPI, a compound that stains the nucleus of cells. 3A shows the control line while 3B,C show the experimental cell lines.

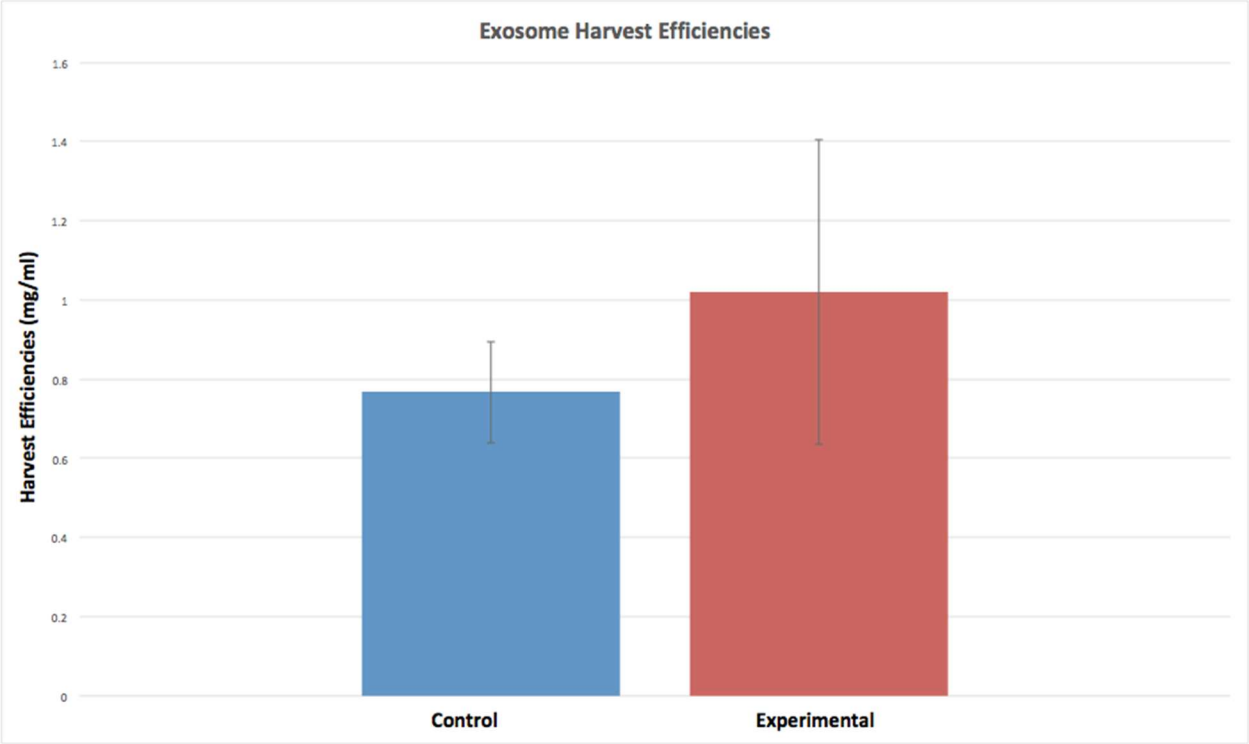

Figure 4: Exosome Harvest Efficiencies. Figure 4 is a graph of our average yields for exosome harvests, done in triplicate. There is no significant difference in the control and experimental yields ($p > 0.05$).

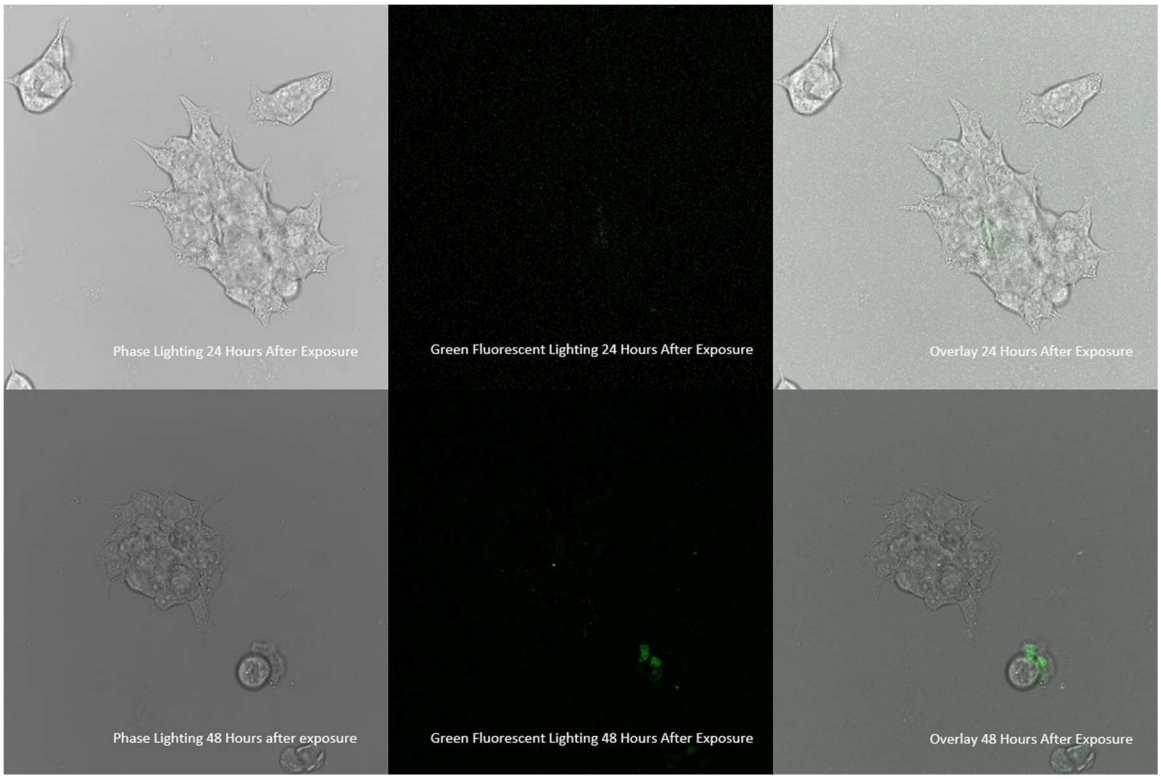

Figure 5: Exosome Uptake Experiment. Figure 5 displays our exosome uptake experiment. In this experiment we exposed regular HEK 293 cells to 100µl of exosomes to ensure that they could still be uptaken. We monitored the cells for 48 hours after initial exposure. 5A,C show the cells in phase lighting while 5B,E show them under green fluorescent lighting at 24 and 48 hours respectively. 5C,F show an overlay at each of those timepoints. In 5F you can see that 48 hours after exposure there was a fair amount of uptake into the cells.

Discussion

In Figure 1, the confocal microscopy pictures taken 31 days after transfection confirms the initial success of the transfection protocol. There is a strong GFP signal in the pictures, and the overlays in Figures 1C and 1F show that the fluorescence is coming from the cells themselves.

Although the confocal microscopy pictures in Figure 1 confirmed the initial success of the transfection, they did not confirm the successful integration of the engineered vector into the genome of the cells themselves, which would indicate a stable cell line. In Figure 2, which depicts more confocal microscopy pictures taken 79 days after transfection, the cells are still stably producing GFP, which confirms the integration of the vector into the genome and validates our stable cell lines.

After validating the stable cell lines, the next step was to ensure the GFP signal was coming from the engineered exosomes and was not being generated in any other part of the cell. This verification was done through the use of DAPI staining, which is a blue fluorescent stain that binds to DNA and stains the nucleus. Since exosomes are produced in the cytosol of the cell, the expectation is that the GFP signal is coming from only the cytosol and not the nucleus. Comparing the GFP and the DAPI fluorescence could reveal precisely where the individual signals were coming from. This is shown in Figure 3B and 3C, where the two engineered cell lines have green and blue fluorescence that is not overlapping. This lack of overlap shows that the exosomes are responsible for the GFP signal and also that they have not entered the nucleus, which is not seen in control exosomes.

Figure 4 contains the average harvest yields for the engineered and the control exosomes. Each condition had three harvests, with the average $\pm$ the SEM shown. There is no significant difference between the harvest yields of the two conditions. This validates one of the main goals of the project, which was to be able to produce engineered exosomes with the same cells for an indefinite period of time. The similar yields from engineered and control cell lines means that the vector we engineered into the exosomes did not significantly affect their production.

Lastly, the ability of engineered exosomes to be taken up by cells needed to be

verified. This was done by isolating and purifying the engineered exosomes, and then reintroducing them into a plate with control HEK cells. If the control cells could take up the engineered exosomes, then this proves that the engineered antigen did not make the exosomes too big to cross cell barriers, which would be an integral part of any future exosome treatment. In Figures 5A, B, and C, the exosomes are in solution but not being taken up by the cells 24 hours after reintroduction to control cells. However, at 48 hours after reintroduction, in Figure 5F, exosomes expressing GFP are inside the cytosol of the control cells. This proves that not only are the exosomes still expressing the vector in their genome, but also that the antigen did not affect their uptake into control cells.

Summary and Conclusion

Project Objectives

Overall, our project focuses on addressing a widespread disease called leukemia, a type of blood cancer which impacts thousands of people each year. Although several current treatments exist, they lack specificity and targeting capabilities, thus resulting in a wide range of debilitating side effects. In order to combat these challenges, our project deals with naturally occurring living nanoparticles called exosomes. The membrane proteins found on the surface of exosomes allow for exosomes to be engineered to have a high binding affinity for leukemia cells. Researchers have developed the Chimeric Antigen Receptor (CAR), which has been proven to bind to proteins found on the surface leukemia cells. Our goal is to prove that a vector of CAR DNA can be integrated into the cell's genome to produce CAR expressing exosomes without disrupting the cell's normal production level of the exosomes. These engineered exosomes can be used to deliver therapeutic drugs specifically to leukemia cells.

Project Design Process

Much of our project design process focused on figuring out what types of assays we wanted to utilize. Prior to senior year, we determined the DNA vector that we were going to use and decided upon integrating the DNA via transfection; however, we were unsure of how to quantify our data to prove our experiment was successful. The main way we decided to accomplish this was through monitoring GFP expression via confocal microscopy. Because GFP is one of the components of our CAR vector, it can be used to show that the transfection was successful and that our cell line now produces exosomes that express the CAR protein. Furthermore, we needed to verify the GFP signal originated from the engineered exosomes and nowhere else in the cell. We decided to employ DAPI staining, a blue fluorescent stain that binds to DNA and stains the nucleus, to identify the nucleus of the cells. Ideally, there should be no overlap between the GFP

and the blue-stained nucleus because exosomes are produced in the cytosol. Moreover, we wanted to prove that the transfection or presence of CAR did not interfere with the cell's normal exosomes yield. We chose to validate this by harvesting exosomes from both the control and experimental cell lines and comparing the values. Finally, we wanted to demonstrate that the engineered exosomes could be reuptaken when introduced back into a cell line. This is a crucial part for confirming that our innovation has potential as a drug delivery method in the future.

Accomplishments

Overall, our results were as expected and our experiment was a success. We were able to transfect the cells with CAR DNA. We established a stable cell line by passaging and changing the cell media biweekly for over 3 months after transfection. This is a noteworthy milestone because a single mishap or contamination would nullify our research and force us to start over. The expression of GFP via confocal microscopy after this time verifies that the DNA had successfully been integrated into the genome of the cells. In addition, the DAPI staining procedure proved that exosomes were present in the cytosol of the cell as one would expect. Next, we found no significant difference between the exosome harvest efficiencies in the control and experimental control lines. This shows that the introduction of CAR does not inhibit the cell's ability to generate exosomes. Lastly, we verified that the engineered exosomes could be reuptaken back into a cell line, which is essential in proving that exosomes could serve as a viable drug delivery option. In conclusion, our design worked and our project was successful. We believe that the CAR expressing exosomes could feasibly be explored as an option to effectively treat leukemia in the future.

Future Work

In the future, one could send out the engineered exosomes to a third party company for analysis. This analysis would elucidate features of the exosomes that were

beyond the capacity available at Santa Clara University such as the size of the exosomes and more accurate harvest values.

The next step would be to conduct an experiment in which the affinity of the engineered antigen itself could be measured. To accomplish this, one would need cells expressing the specific biomarker on leukemia cells. One could ten put both the leukemia biomarker cells as well as control HEK cells onto the same plate and introduce the engineered exosomes into the plate, similar to the experiment conducted in Figure 5. After, one could use confocal microscopy to visualize whether or not the engineered exosomes were preferentially uptaken by the leukemia cells over the control cells. This would give valuable insight into how the engineered exosomes would act in the human body, where there are many different cell types in the same area.

The two stable cell lines that we engineered were cryopreserved for potential future work. If another group of students were to continue this project, they would have a significant time advantage to perform verification experiments, a stable cell line expressing the vector has already been established.

Lessons Learned

During the course of this year-long project, there were many aspect where our group thrived, and other shortcomings that we could have improved upon. There were things we could have done both before beginning the project and during the project itself that could have set us up for greater success. These follow, in no particular order.

One way we could have improved was better planning prior to the senior design process. As a group, we were somewhat unprepared and did not have a clear picture of what our senior design project would be about until the end of junior year. This did not allow us to take specific courses that could have expedited our learning, which meant that we had to spend valuable project time learning the skills that could have been learned earlier. Specifically, our project involved a great deal of cell culture techniques. If we all had taken the cell culture lecture and laboratory prior to starting our project, we could have eliminated much of the learning curve in the initial stages of the project. This meant

that the back end of the project was cramped on time and we could not perform as many verification experiments as we would have liked.

Engineering Standards and Realistic Constraints

Various engineering standards and realistic constraints impact our project and should be considered. First, our design leads to some economical constraints. Producing CAR-expressing exosomes is a costly task that requires the use of high-quality, specialized equipment. For example, simply maintaining the exosome producing cells requires the use of microscopes, incubators, aspirators, centrifuges, pipetman, and micropipettes in addition to the necessary cell media. Therefore, the cost of production is quite high, which will likely drive up the price for the consumer and can lead to some ethical dilemmas.

If the cost is high, patients of lower socioeconomic status may not be able to afford the treatment they need to fight Leukemia, while members of the upper class can afford the treatment. This results in money potentially serving as the deciding factor for who lives or dies, which is undoubtedly an ethical concern. In addition to monetary ethical issues, our project uses HEK 293 cells (STEM cells) as the basis for our experiments, which are somewhat controversial. HEK 293 cells are derived from human embryonic kidneys cells and are grown in tissue culture. The use of human embryonic stem cells is controversial because human embryos are destroyed to harvest the cells and the question of when life begins is highly debated.

Furthermore, manufacturing serves as another constraint because producing CAR-expressing exosomes is a tedious tasks that requires a great deal of time and effort. Harvesting exosomes is a long process that is quite laborious. Also, cell lines must be constantly maintained and sustained through passaging and changing media. Manufacturing time and exertion is a significant constraint that must be considered in our project.

Moreover, sustainability is another engineering standard that should be addressed. After the CAR-expressing exosomes are harvested from the engineered HEK 293 cell, they are not very sustainable and will not last long on their own. The exosomes must be

either used quickly for treatment or frozen down. Even though the exosomes themselves are not sustainable, the cells which produce the exosomes are sustainable and can be maintained. This help increase the sustainability of our project.

Lastly, our experiment does raise some environmental issues. Due to sterile technique, each pipet and aspirating tip can only be used once before being discarded. This results in the accumulation of an abundance of plastic waste from experiments. For example, each passage uses 3 aspirating tips, 4 pipets, a well plate, a centrifuge tube, as well as a pair of gloves for each person working. Although this may seem wasteful, it is a sacrifice we must make in order to prevent cell contamination.

Resources

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Appendix A: Design

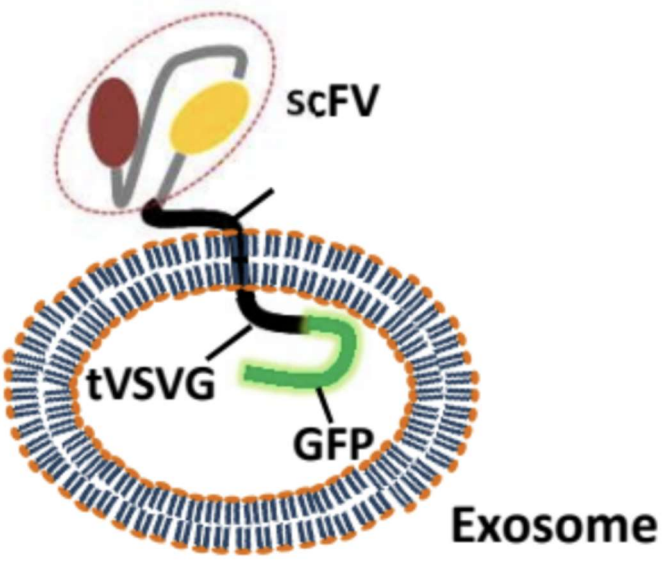


Figure 6. Design of CAR-Expressing Exosome

Figure 7. Expression Vector

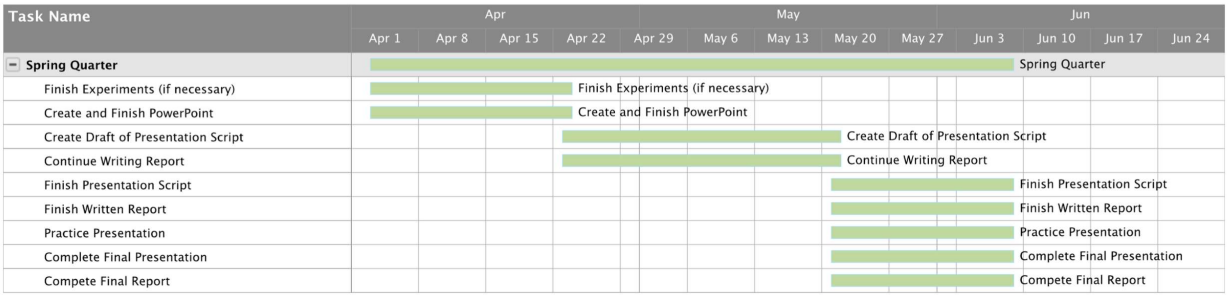
Appendix B: Gantt Charts

Table 3: Gantt Charts

Senior Design Project Timeline (Fall)

Senior Design Project Timeline (Winter)

Senior Design Project Timeline (Spring)



Appendix C: Final Presentation Slides

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Production of Engineered Living Nanoparticles for Targeted Blood Cancer Therapy

Peter Mitchell
Michael Pierotti
Matthew Piro

Advisor: Dr. Bill Lu

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Blood Cancer - Leukemia in the US

- 65,000 new cases every year
- 350,000 people living with Leukemia
- 24,500 deaths caused by Leukemia every year

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Current Cancer Therapies

- Chemotherapy
- Radiation Therapy
- Biological Therapy

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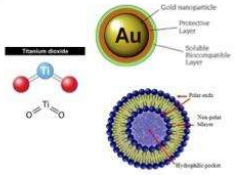
Nanoparticles

- Gold
- TiO_2
- Liposomes



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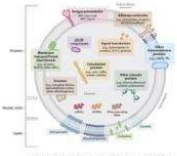
Our Solution: Exosomes

- Biocompatible
- Able to penetrate tissue
- Membrane proteins



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Our Innovation

- Modify Exosomes to express CAR
- Utilize GFP Monitoring

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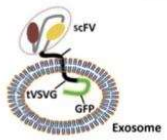
Design



- Chimeric Antigen Receptor (CAR)
- scFV: single-chain variable fragment
- tVSVG: truncated VSVG protein
- GFP: Green fluorescent protein

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Molecular Implementation

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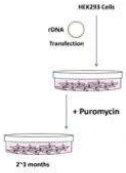
Procedure



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Significance of our Innovation

- Safer than current cancer therapies
  - More effective
  - Reduces side effects
- Foundational
  - Applicable for other malignancies

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Accomplishments

- Established stable cell line
- Confocal pictures
- Isolated and purified exosomes
- Exosome delivery experiment

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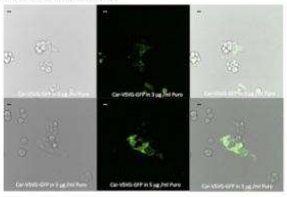
Initial Success

Cell Line 1

Cell Line 2



Day 31

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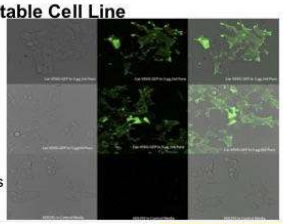
Stable Cell Line

Day 79

Cell Line 1

Cell Line 2

Control Cells



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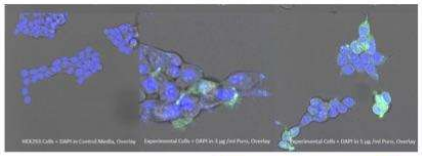
DAPI Staining

Control Cells Cell Line 1 Cell Line 2



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Exosome Isolation + Purification

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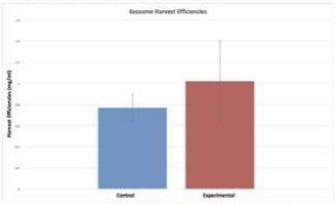
Exosome Harvest Yields



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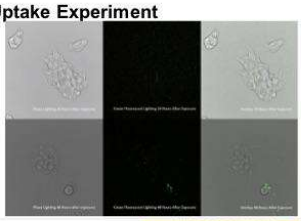
Uptake Experiment

24 hours

48 hours



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Future Plans for Project

- Send out exosomes for characterization
- Work with leukemia cells

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Thank You!
Questions?
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Appendix D: Budget

Table 4: Detailed Budget

Materials Budget (Complete Funding by Undergraduate Programs):

- 293T packaging cells (ATCC # CRL-11268) — Supplied
- Prepared plasmid DNA for: — $600.00
  - CAR-t scFV
  - tVSVG
  - GFP
- PEI: — $390.00
- DMEM media (ATCC #30-2002): — Supplied
- OptiMem Reduced Serum Media (Invitrogen # 31985-070): — Supplied
- FBS (ATCC #30-2020): — Supplied
- Pen/Strep (ATCC #30-2300): — Supplied
- 15mL Centrifuge Tubes (Fisher # 05-538-53F): — Supplied
- 50mL Centrifuge Tubes (Fisher # 07-203-510): — Supplied
- Qiagen Miniprep Kit: — $400.00
  - Total: — $1,390.00
  - Funding Received — $1,500.00
  - Remaining Budget — $110.00